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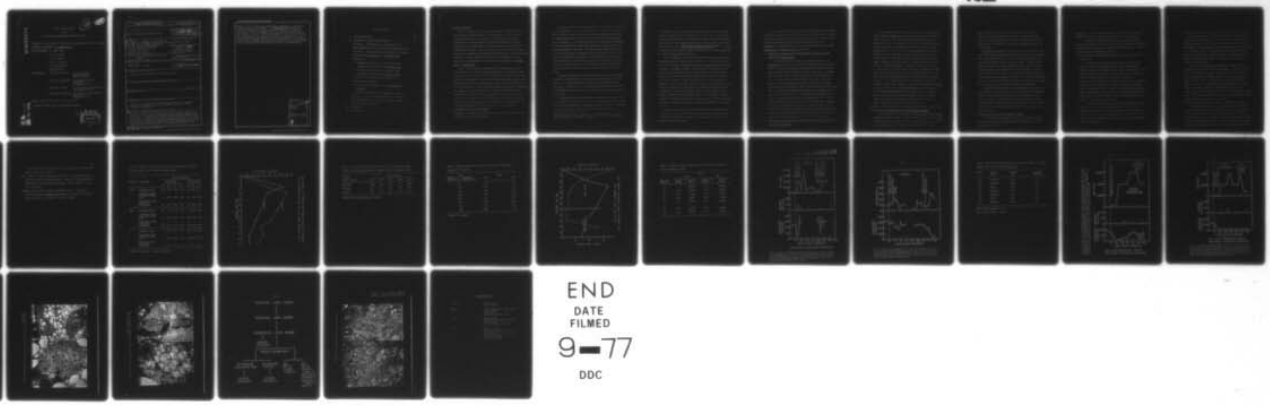

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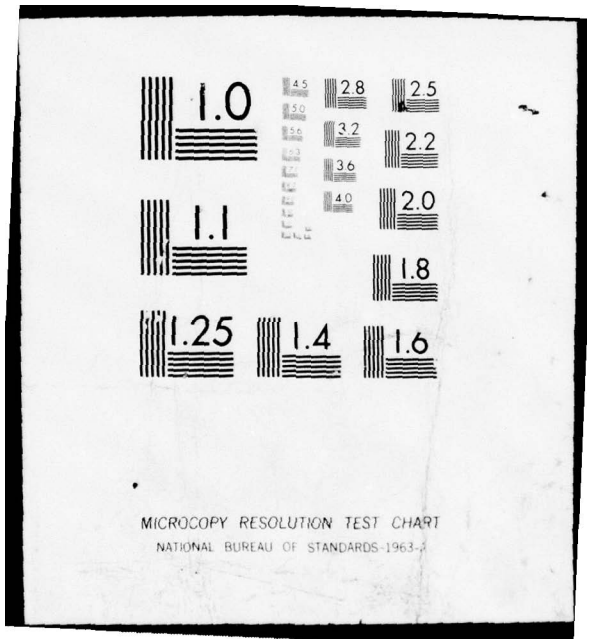
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ANNUAL PROGRESS REPORT

TO THE

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND

CONTRACT: U.S. Army DADA 17-70-C-0044--Control of Hemotropic Diseases of Dogs

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Tropical canine pancytopenia; Ehrlichia canis; babesiosis; leukopenia; Rhipicephalus sanguineus; platelets, migration; epistaxis; indirect fluorescent antibody test

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

A platelet migration inhibition (PMI) test was developed for detection of serum antiplatelet activity in experimentally and naturally induced canine ehrlichiosis. Examination by scanning electron microscopy (SEM) of platelets treated with normal serum and serum having inhibitory activity revealed that uninhibited platelets have numerous pseudopod formations whereas inhibited platelets are generally rounded, smooth, and occasionally have membrane damage and apparent shrinkage and loss of intracellular contents. The

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potential of the brown dog tick Rhipicephalus sanguineus as a reservoir of Ehrlichia canis was investigated. R. sanguineus adults harbored and efficiently transmitted E. canis to susceptible dogs for as long as 155 days after detachment as engorged nymphs from a dog experiencing acute ehrlichiosis. Two modifications of the original tissue culture technique for the propagation of E. canis were developed to study the effect of serum and macrophages from infected dogs on growth and development of E. canis and to provide continuous production of large quantities of E. canis antigen. A combination of chemotherapy and serologic monitoring of disease was found to be a useful method for field control of tropical canine pancytopenia.

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I. GENERAL INFORMATION

From its inception some 6 years ago, the project was problem-oriented toward acquiring knowledge needed for the development of control measures for canine ehrlichiosis and babesiosis. The project was conducted as a collaborative venture between this station and the Division of Veterinary Medicine, Walter Reed Army Institute of Research and its overseas activities in Thailand and Malaysia. These collaborative efforts have elucidated many aspects of the epidemiology, pathogenesis, and immunology of canine ehrlichiosis. In addition, valuable information was obtained on serodiagnosis of canine babesiosis and understanding of the effect of dual infections with Ehrlichia canis and Babesia canis.

Some of the more specific accomplishments in ehrlichiosis have been: (1) Development of a method for in vitro cultivation of the causative agent; (2) development of a serologic test for detection of ehrlichiosis under field conditions; implementation of the test has helped determine the incidence and devise control measures for ehrlichiosis in certain areas overseas and in the U.S.; (3) definition of ultrastructural characteristics and mode of development of E. canis in the vertebrate hosts; (4) evidence by means of radioactive tracers that thrombocytopenia in E. canis-infected dogs is the result of increased platelet destruction which begins within a few days after infection; (5) evidence that Ehrlichia equi, the causative agent of equine ehrlichiosis, causes experimental infections in dogs, cats, and nonhuman primates.

II. OBJECTIVES OF THE LAST YEAR OF SUPPORT

Objective 1. Investigation into Immunologic Basis of Early Thrombocytopenia in Tropical Canine Pancytopenia (TCP).

Pancytopenia, particularly thrombocytopenia, is the characteristic hematologic manifestation of TCP. An earlier study of this group using radioactive tracers has shown that severe reduction in platelet population is due to increased destruction of these cells; however, the underlying mechanism was not determined. It is well-established that sensitization of lymphocytes in an animal infected with intracellular parasites may occur within 2 to 3 days after exposure to the agent. The study conducted during the past year of support has examined the immunologic basis of pancytopenia in TCP. This first report demonstrates the existence of monocytotoxic effect exerted by lymphocytes from a dog infected with E. canis. To avoid histocompatibility effects, the study was conducted in an autologous system with lymphocytes (effector cells) and monocytes (target cells) being derived from the same animal.

Monocytes were prepared and cultured according to the method previously described in this laboratory.[1] The cells were cultivated for 7 to 10 days prior to use. Optimal conditions for labeling monocytes with ^{51}Cr were established. Monocytes ($20 - 30 \times 10^6$) were incubated with 200 µCi of ^{51}Cr in RPMI medium at 37° C for 1 hour. The cells were washed 3 times to remove excess label.

Autologous lymphocytes were separated on Ficoll-Hypaque gradient. The optimal effector cell-target cell ratio was determined by studying the effect of varying the concentration of lymphocytes relative to monocytes. This ratio was found to be 100 lymphocytes to 1 monocyte and was used throughout

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[1]Myindo, M. B. A., Ristic, M., Huxsoll, D. L., and Smith, A. R.: Tropical Canine Pancytopenia: In vitro Cultivation of the Causative Agent-Ehrlichia canis. Am J Vet Res, 32 (1971): 1651-1658.

the cytotoxicity test. Triplicate cultures of labeled monocytes and lymphocytes at optimal ratios were incubated at 37° C for 18 hours. After centrifugation the radioactivity in the supernatant fluid was counted and expressed as a percentage of the expected total release. Thus, % specific cytotoxicity (^{51}Cr release) = $100 \times \frac{^{51}\text{Cr released by test—spontaneous release}}{\text{total release—spontaneous release}}$. The effect of immune serum was studied by adding varying dilutions of anti-*E. canis* serum to optimal monocyte-lymphocyte mixtures.

Table 1 summarizes the results on release of ^{51}Cr from monocytes following interaction with lymphocytes from dogs with TCP. The base line at 0 day was 5.5% ^{51}Cr release. Following infection with *E. canis*, lymphocytes became cytotoxic to autologous monocytes. The earliest day of testing was day 10 after infection which coincided with maximal cytotoxicity by lymphocytes of dog 2135. Dog 2136 showed the highest cytotoxicity at day 15, and dog 7 at day 31 after infection. Figure 1 shows the kinetics of the monocytotoxic response in dogs 2135 and 2136. The effect of various concentrations of anti-ehrlichia antibodies on monocytotoxicity of lymphocytes from dogs with TCP was examined. There was no significant effect of these antibodies (with or without complement) on monocytotoxicity (Table 2). The degree of monocytotoxicity was directly proportional to the concentration of lymphocytes relative to monocytes indicating that immune lymphocytes were effectors of cytotoxicity (Table 3).

The monocytotoxic effect had an apparent temporal relationship with the thrombocytopenia in that the monocytoxic effect closely paralleled the drop in thrombocyte count (Fig. 2, Table 4). As thrombocyte levels returned to normal, cytotoxicity decreased.

It was concluded that dogs with acute TCP showed lymphocyte-mediated monocytotoxicity within an autologous system. This monocytotoxic effect subsided with clinical remission of TCP. Cytotoxicity appears to be antibody and complement independent and proportional to monocyte-lymphocyte ratio.

Objective 2. Immune Responses in Horses and Dogs Following Infections with Ehrlichia equi and Ehrlichia canis, respectively.

A. Measurement of humoral and cell-mediated immune responses in ponies infected with Ehrlichia equi.

The immune response of 6 ponies to experimental infection with Ehrlichia equi was studied with respect to cellular and humoral activity. Primary inoculation of ponies with blood derived from acutely infected ponies produced clinical ehrlichiosis between 5 and 8 days. Inoculation with convalescent blood or organ homogenates of ponies recovered from the acute disease failed to induce disease (Figs. 3, 4). Only ponies showing signs of ehrlichiosis upon primary inoculation with E. equi developed high and long-lasting indirect fluorescent antibody (IFA) titers and cell-mediated (CMI) responses as measured by the peripheral blood leukocyte migration (LMI) test. Cell-mediated immune responses fell to a base line (preinoculation) level by approximately day 150. The IFA titers were usually high in the early periods of observation. Challenge inoculation of ponies which recovered from clinical disease and their treatment with tetracycline failed to induce clinical ehrlichiosis. This study suggests that following clinical ehrlichiosis, ponies become protected to challenge inoculation by a state of sterile immunity.

B. Measurement of humoral and cell-mediated immune responses in dogs infected with Ehrlichia canis.

Twelve German shepherds and 5 beagles of both sexes were experimentally infected with Ehrlichia canis and their cell-mediated and humoral immune responses to this organism were studied during an observation period of 120 days. It was found that 58.33% of the German shepherds had positive LMI test results using blood leukocytes while 80% of beagles became positive. Five out of the 12 German shepherds developed severe chronic TCP and had lower CMI responses than dogs with the nonsevere disease (Table 5). Both breeds of dogs developed high IFA titers to E. canis. Treatment of infected dogs with antilymphocyte serum (ALS) did not result in detectable harmful or beneficial effects (Figs. 5, 6, 7) in either breed of dog in the course of infection. However, some transitory fluctuation of platelet counts was noted following treatment. The beagle and some German shepherds mounted a strong CMI response against E. canis. It is suggested that this cellular response contributes to protection against severe chronic TCP. Data did not suggest that E. canis infection induces a state of immunosuppression to other antigens, i.e., 2,4-Dinitrochlorobenzene (DNCB) and Old Tuberculin (OT). A specific fraction was extracted by column chromatography from media in which sensitized lymphocytes were activated by antigen and its ability to inhibit leukocyte migration was demonstrated (Fig. 8).

Objective 3. Host-Parasite-Vector Relationship in Ehrlichiosis and Babesiosis.

A. Development of E. canis in the tick Rhipicephalus sanguineus.

Certain aspects of the development of Ehrlichia canis in Rhipicephalus sanguineus ticks were studied. It was found that prior feeding of E. canis-infected nymphs was a desirable, if not necessary, preliminary treatment for successful infection of dogs with macerated ticks. It remains

unclear whether tick feeding increased the number or altered the virulence of ehrlichiae within tick tissues. The phenomenon may be similar to rickettsial "reactivation" whereby feeding by carrier ticks stimulates the transformation of avirulent R. rickettsii organisms into virulent forms.

Ehrlichia canis organisms were detected by immunofluorescent microscopy in the midgut and homocytes and by electron microscopy in the midgut and salivary glands (Figs. 9, 10, 11, 12) of partially engorged adult ticks which had been infected as larvae and nymphs. Organisms were not observed in the ovary. Intracytoplasmic inclusions contained 1 to 80 elementary bodies, each surrounded by 2 distinct membranes. Infection of the midgut and salivary gland was confirmed by injecting homogenates of these tissues into susceptible dogs (Fig. 13). Staining of gut smears of partially engorged adult ticks by fluorescein-conjugated anti-E. canis antibody was found to be a reliable indicator of the infection.

Symbiotic rickettsiae (Figs. 14, 15) morphologically distinct from ehrlichia were found in tick ovaries. Data derived in the present study support earlier findings that transovarial tick transmission does not occur in E. canis. In epidemiological terms, this means that carrier dogs remain the principal source of E. canis in nature. However, recent studies in our laboratory demonstrated tick transmission from acutely infected dogs only.

B. Concurrent infections with E. canis and B. canis.

It was found that concurrent infections of E. canis and B. canis were marked by more severe signs than those observed in dogs infected with each agent alone.

Objective 4. Control of Canine Ehrlichiosis and Babesiosis in Thailand, Malaysia, and the U.S., and Public Health Relevance of These Diseases.

Collaborative research studies conducted by performing indirect fluorescent antibody tests for canine ehrlichiosis and babesiosis contributed to the epidemiology and control of outbreaks of these diseases. U.S. Army units involved in collaborative efforts are:

1. Medical Component SEATO Laboratory, Bangkok, Thailand, c/o LTC David E. Davidson, Jr.

   In an epizootic of canine ehrlichiosis among 316 military dogs (Pakchong area), 161 serologically positive cases were identified. Fifty-four of these dogs exhibited clinical signs of the disease. Other serologic studies of ehrlichiosis among pet dogs in widely separated regions of Thailand suggest that the disease is endemic in
   The epizootic is being controlled by the elimination of ticks, serologic identification and treatment of carriers with 30 mg tetracycline per pound body weight per day for 14 days, and by continuous daily prophylactic administration of 3 mg tetracycline per pound body weight per day. Implementation of control measures caused serologic remission in two-thirds of the cases during the past year.

   Other studies in Thailand concerned serologic examination of more than 100 German shepherd dogs belonging to the U.S. Air Force unit stationed at Udorn base. This unit was in the process of relocation to Okinawa and it was mandatory that diseased dogs not be moved to a new location. Initial examination revealed 15 positive dogs and additional tests are under way.

2. Medical Research Unit, Kuala Lumpur, Malaysia, c/o LTC David L. Huxsoll.

Malaysian Army dog wing - Pulada, Johore, Malaysia, other military installations, and civilian dogs have been screened for ehrlichia and babesia antibodies. Among 130 dogs, 23 babesia and 2 ehrlichia reactors were detected. Anemia noticed in these dogs was apparently due to babesiosis. Another 112 samples of Thai military and police dogs received by way of Malaysia revealed 26 ehrlichia reactors. Epidemiologic studies aimed at establishing the incidence of ehrlichiosis and babesiosis in Malaysia is being continued.

During the last decade, babesiosis has been recognized to affect human beings and cause severe disease and fatality. Thus far, 14 cases of the disease have been reported. A number of cases of nonspecific fever among military and civilian personnel have also been reported in Malaysia by LTC Huxsoll. Examination for babesia antibodies of 213 human sera from that country revealed 15 positive samples. These sera originated from padi planters, a maternal-cord blood study, P.U.O. Gombak Hospital, and the Bukit Lanjang area. Future plans of this program are to attempt to isolate babesia from the blood of serologically positive patients using animals such as hamsters and dogs.

3. Division of Veterinary Medicine, WRAIR, c/o LTC Edward H. Stephenson.

LTC Stephenson has served as a coordinator of the above-described overseas programs as well as the principal investigator of epidemiologic and serodiagnostic studies of canine blood disease among military and civilian dogs in the U.S. The largest study conducted in the U.S. during the past year concerned an outbreak of ehrlichiosis among dogs in Phoenix, Arizona. In a population of 514 dogs, 114 were serologically positive

for ehrlichiosis. More than 2 dozen of these dogs were treated in a Phoenix animal hospital for varying degrees of epistaxis and other clinical signs of ehrlichiosis.

A military dog buying team from Lackland AFB purchased 54 dogs from the Phoenix area for military use and shipment to Kadina AFB, Okinawa. Serologic examination of 124 dogs from Lackland revealed 21 positive; 11 of these reactors were among dogs purchased from the Phoenix area. The study of canine ehrlichiosis epizootic in the Phoenix area and its relevance to the purchase of military dogs is being continued.

4. Division of Experimental Pathology, c/o LTC Paul K. Hildebrandt.

Autopsies for histopathologic and electron microscopic examination of tissue lesions in horses infected with Ehrlichia equi were performed at this station by researchers from the Division of Experimental Pathology at WRAIR. Examination of these tissues is now in progress.

Additional cases of canine ehrlichiosis were identified serologically in the following states; Arizona, 5; California, 1; Florida, 5; Georgia, 10; Illinois, 3; Maine, 1: Massachusetts, 2; Ohio, 2; Texas, 5, and Virginia, 1.

III. CONCLUSIONS

Investigation into the immunopathologic basis of early pancytopenia in TCP revealed that activated lymphocytes from an infected animal exert a cytotoxic effect on autologous monocytes. The underlying mechanism could be an E. canis-related membrane alteration of infected and noninfected monocytes. Cytotoxicity appears to be antibody and complement independent. Studies in progress concern investigation of the possible existence of a similar immunopathologic mechanism affecting blood platelets, the destruction of which seems to constitute the

most pronounced patho-physiologic lesion in this disease.

Cell-mediated immunity was demonstrated in horses and dogs infected with E. equi and E. canis, respectively. A recovery from the acute phase of infection in horses is followed by sterile protective immunity, while in dogs this immunity depends upon persistence of the organism in "carrier dogs."

Study of the development of E. canis in Rhipicephalus sanguineus ticks revealed the presence of the organism in the midgut and hemocytes by immunofluorescence microscopy and in the midgut and salivary glands by electron microscopy. It was found that prior feeding of E. canis-infected nymphs was necessary for successful infection of dogs with macerated ticks. The phenomenon resembles the "reactivation" principle whereby feeding by carrier ticks stimulates the transformation of avirulent R. rickettsii into virulent forms. The organism was not detected in the ovaries of infected ticks ruling out transovarial tick infections and suggesting that carrier dogs remain the principal source of E. canis in nature.

Collaborative research studies with the U.S. Army Medical Research Unit at the WRAIR, Thailand, and Malaysia have resulted in the development of effective means for recognition and control of canine ehrlichiosis and babesiosis epizootics in the U.S. and abroad.

IV. PUBLICATIONS

Lewis, George E., Jr., Huxsoll, David L., Ristic, Miodrag, and Johnson, Anthony J.: Experimentally Induced Infection of Dogs, Cats, and Nonhuman Primates with Ehrlichia equi, Etiologic Agent of Equine Ehrlichiosis. Am J Vet Res, 36 (1), 1975: 85-88.

Smith, Ronald D., Small, Erwin, Weisiger, Rita, Byerly, C. S., and Ristic, Miodrag: Isolation in Illinois of a Foreign Strain of Ehrlichia canis,

the Causative Agent of Canine Ehrlichiosis (Tropical Canine Pancytopenia). JAVMA, 166 (2), 1975: 172-174.

Smith, Ronald D., Ristic, Miodrag, Huxsoll, David L., and Baylor, Richard A.: Platelet Kinetics in Canine Ehrlichiosis: Evidence for Increased Platelet Destruction as the Cause of Thrombocytopenia. Infect & Immun., 11 (6), 1975: 1216-1221.

Weisiger, Rita M., Ristic, Miodrag, and Huxsoll, David L.: Kinetics of Antibody Response to Ehrlichia canis Assayed by the Indirect Fluorescent Antibody Method. Am J Vet Res, 36 (5), 1975: 689-694.

Table 1. Release $^{51*}Cr$ from monocytes following interaction with lymphocytes from dogs with TCP within autologous system.

Dog. No	Test System	Days Post Infection						
		Acute Phase				Chronic Phase		
		0	10	15	31	40	45	53
2135	Lymphocytes alone	5.5*	75.0	46.0	33.5	25.3	22.0	12.1
	Lymphocytes & heat-inactivated immune serum	0	80.0	42.5	50.0	40	22.0	35.0
	Lymphocytes & fresh immune serum (complement)	3.5	76.5	43.0	48.5	38.0	35.0	36.4
2136	Lymphocytes alone	0.5	45.6	81.5	64.0	53.0	36.0	40.0
	Lymphocytes & heat-inactivated immune serum	1.0	44.0	78.0	75.0	55.0	34.5	41.6
	Lymphocytes & fresh immune serum (complement)	2.0	48.0	79.5	80.0	49.6	40.0	42.0
7	Lymphocytes alone	0	66.0	55.0	77.0	31.5	30.0	45.5
	Lymphocytes & heat inactivated immune serum	0	49.0	54.5	78.0	36.0	29.5	41.0
	Lymphocytes & fresh immune serum (complement)	0	62.0	52.0	64.5	36.0	29.0	50.0

*Percent ^{51}Cr release average of 3 samples.

FIG.1 RELEASE OF ^{51}CHROMIUM FROM MONOCYTES FOLLOWING INTERACTION WITH LYMPHOCYTES FROM DOGS WITH TCP WITHIN AUTOLOGOUS SYSTEM

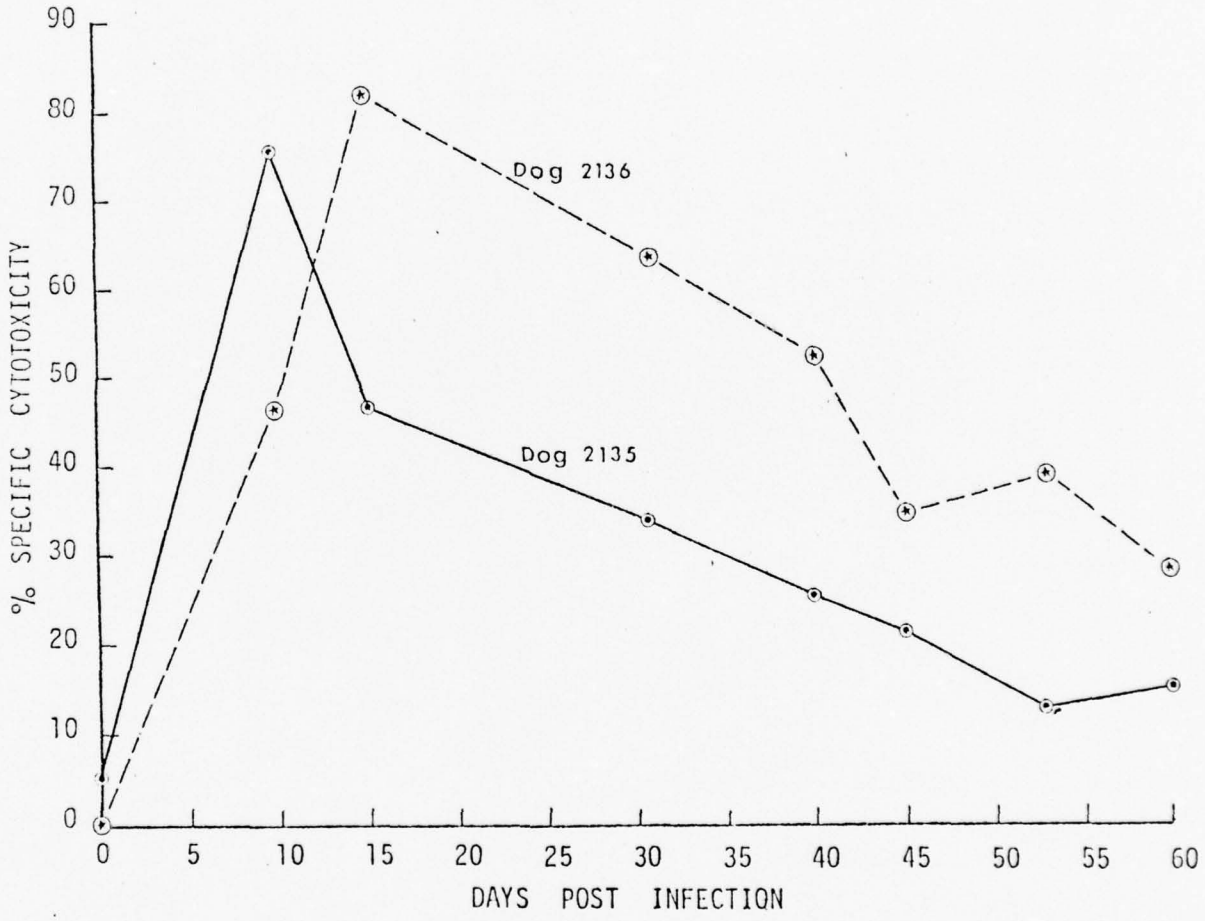


Table 2. Effect of serum dilution on release of ^{51}Cr from monocytes upon interaction with immune "acute phase" lymphocytes within autologous system.

Dog 2135	Serum Dilution				
Source of Serum	1.5	1:20	1:40	1:200	1:800
Autologous	68.0*	64.5	70.0	69.3	69.0
Isologous	74.0	71.3	67.0	73.0	68.6
No serum	72.5	69.6	69.0	70.0	71.0

*Percent ^{51}Cr release average of 3 samples.

Table 3. Effect of target cell - effector cell ratio on cytotoxicity measured by ^{51}Cr release.

Ratio Monocyte (target cell) - Lymphocyte (Effector cell)	Dog No. 2136	2135
1:1	9.5*	12.0
1:10	10.0	7.0
1:20	16.8	19.5
1:40	30.0	26.0
1:60	55.0	45.5
1:80	78.0	65.0
1:100	80.5	75.0
1:200	80.0	77.5

*Percent ^{51}Cr release.

FIG.2 TEMPORAL RELATIONSHIP BETWEEN CYTOTOXICITY TEST AND THROMBOCYTE COUNTS (DOG No.2136)

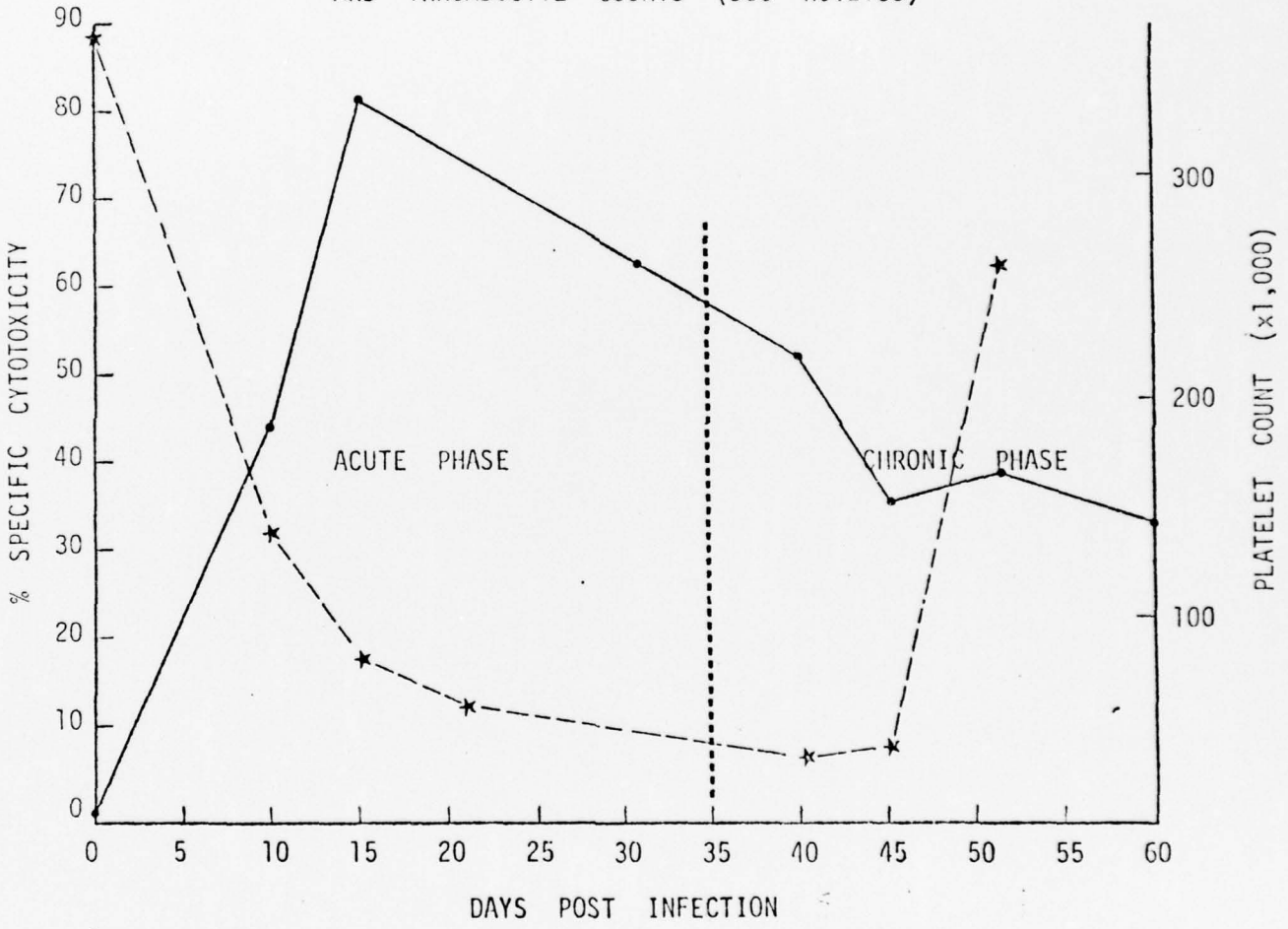


Table 4. Temporal relationship between results of monocyte cytotoxicity test and thrombocyte counts.

Days Post-Infection	Dog No. 2136 Percent ^{51}Cr Release	2136 Platelet Counts	2135 Percent ^{51}Cr Release	2135 Platelet Counts
0	.5	359,000	5.5	400,000
10	45.6	135,000	75.0	260,000
15	81.5	72,000	46.0	29,000
31	64.0	47,000	33.5	23,000
40	53.0	29,000	25.3	260,000
45	36.0	33,000	22.0	300,000
53	40.0	250,000	12.1	296,000

Figure 3

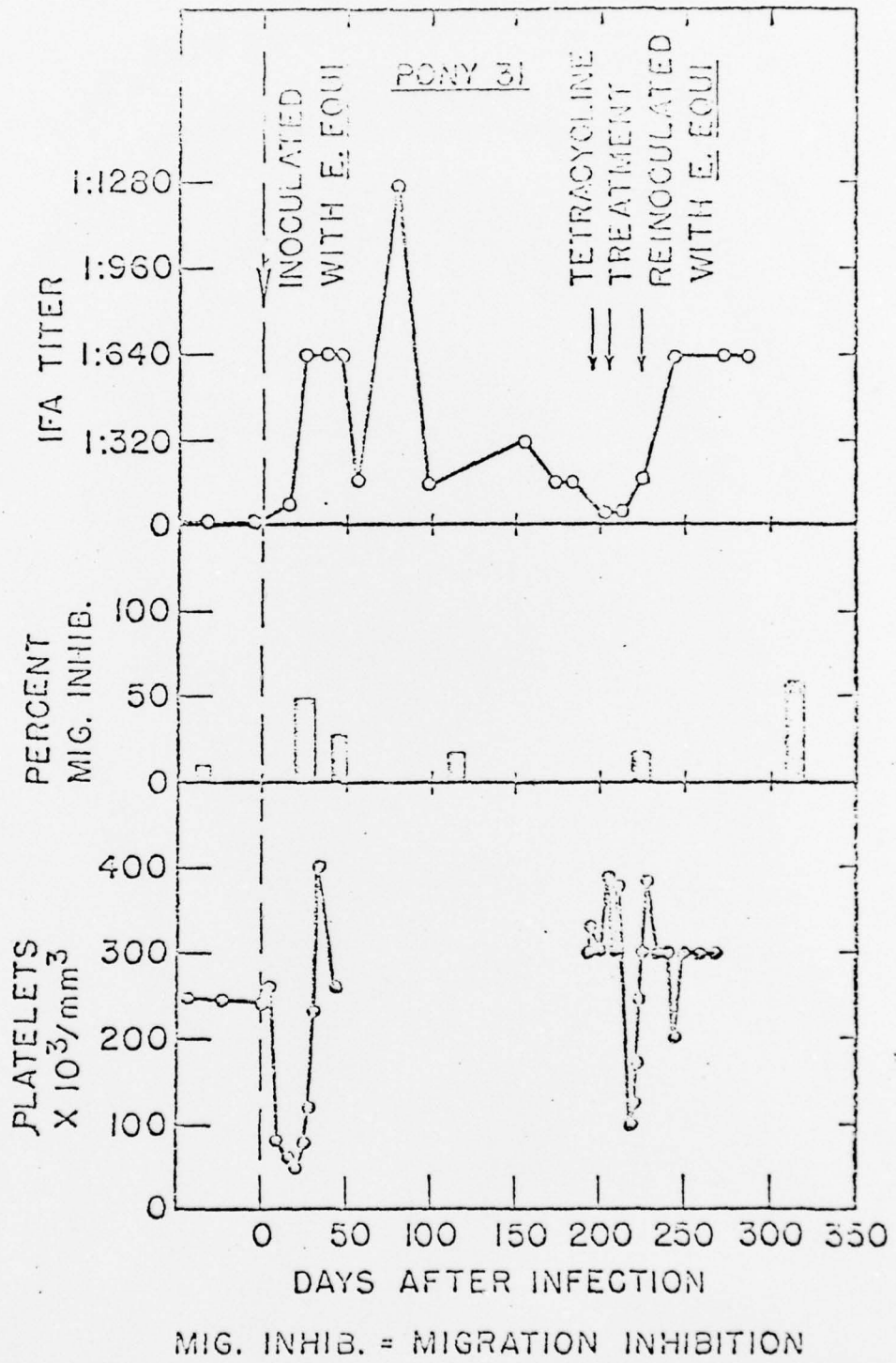


FIG. 3. Pony # 31 inoculated with blood from a pony acutely infected with Ehrlichia equi. The same pony was treated with tetracycline and then reinoculated with E. equi. Note the development of cell mediated immune (CMI) response by the 3rd week and reappearance of the same response after challenge with E. equi.

Figure 4

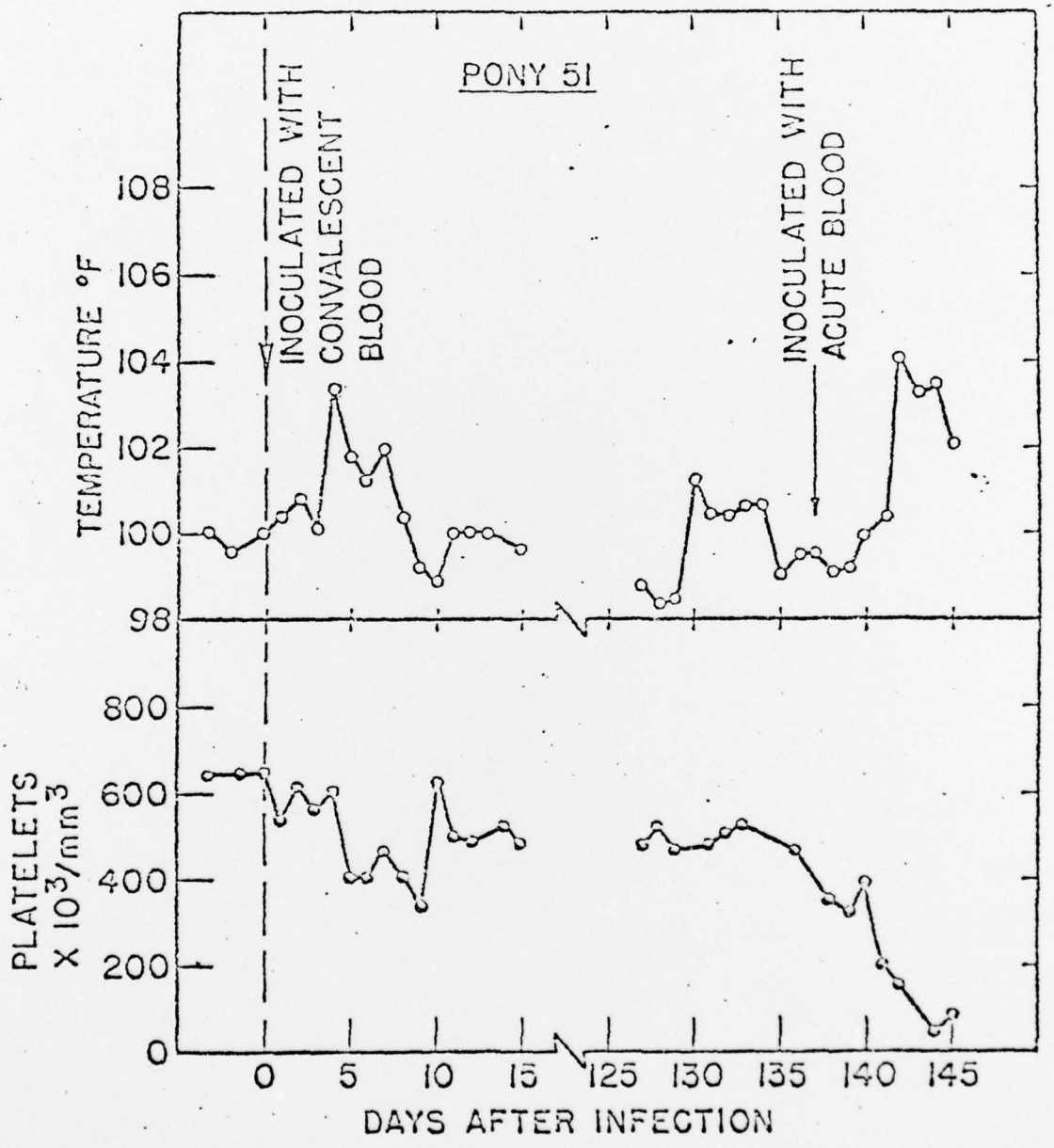


FIG. 4. Effect of inoculation of pony No. 51 with blood derived from a pony convalescing from Ehrlichia equi infection. This inoculation produced fever and mild thrombocytopenia. Reinoculation of the same pony with blood derived from a pony with acute ehrlichiosis produced a high fever and marked thrombocytopenia.

Table 5. Results of the leukocyte migration inhibition (LMI) test in German shepherd dogs infected with *Ehrlichia canis*.

Dog No.	Clinical Disease	Maximum % LMI	Day After Infection
115	Nonsevere	75.0	51
117	Nonsevere	56.60	53
118	Severe	31.60	52
120	Nonsevere	31.10	21
123	Nonsevere	25.0	54
56	Severe	26.0	79
59	Nonsevere	76.0	26

Mean % LMI for nonsevere - 52.7%.

Mean % LMI for severe - 28.8%.

FIG. 5. Leukocyte migration inhibition test, indirect fluorescent antibody (IFA) titer, and thrombocyte counts for German shepherd dog No. 123 that had nonsevere chronic TCP. The effect of treatment with antilymphocyte serum (ALS) is shown. Note the presence of a transitory thrombocytopenia following ALS treatment. Note also that the thrombocyte counts for this dog remained above 50,000/cmm 60 days post infection and before treatment with ALS.

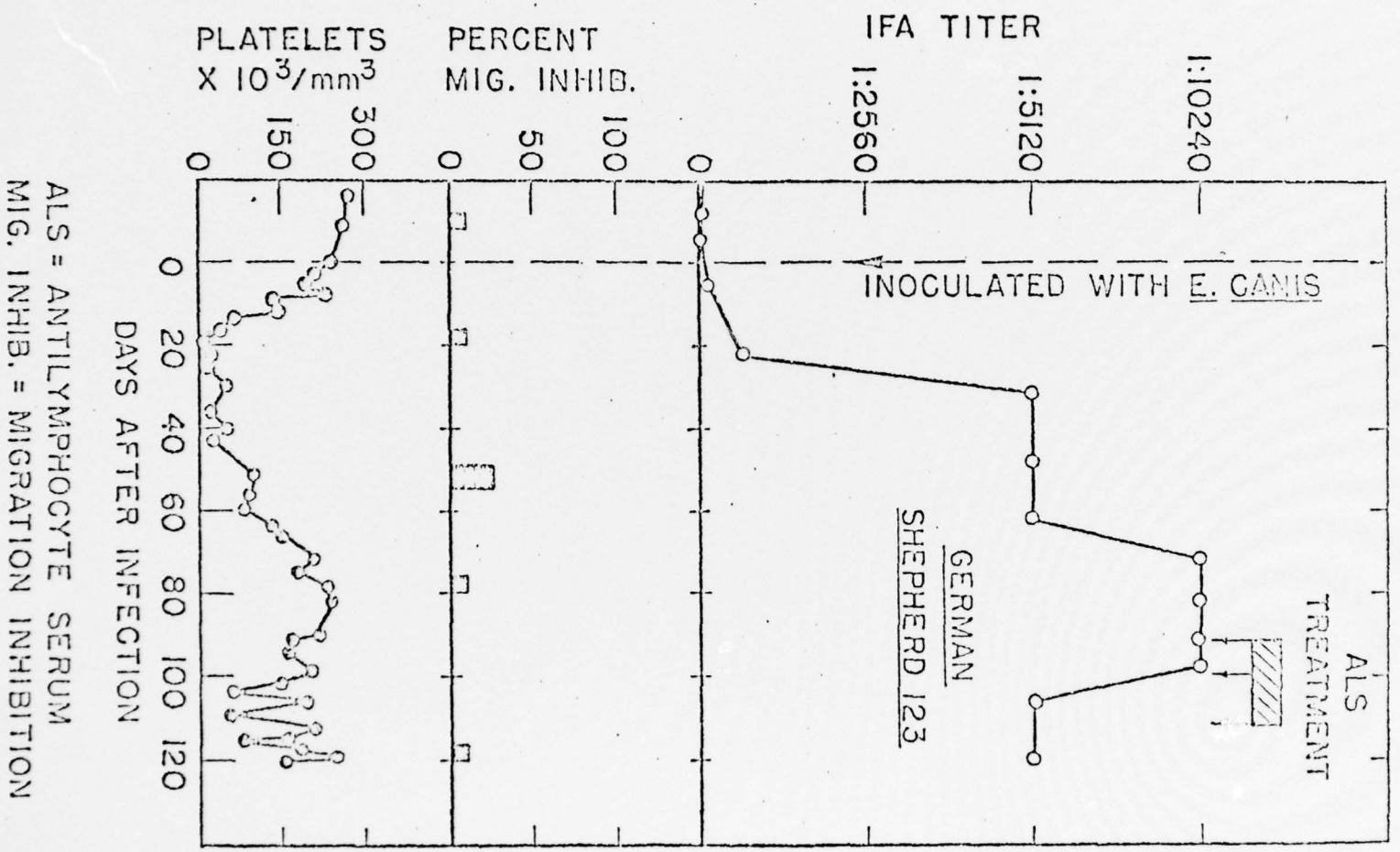


Figure 5

Figure 6

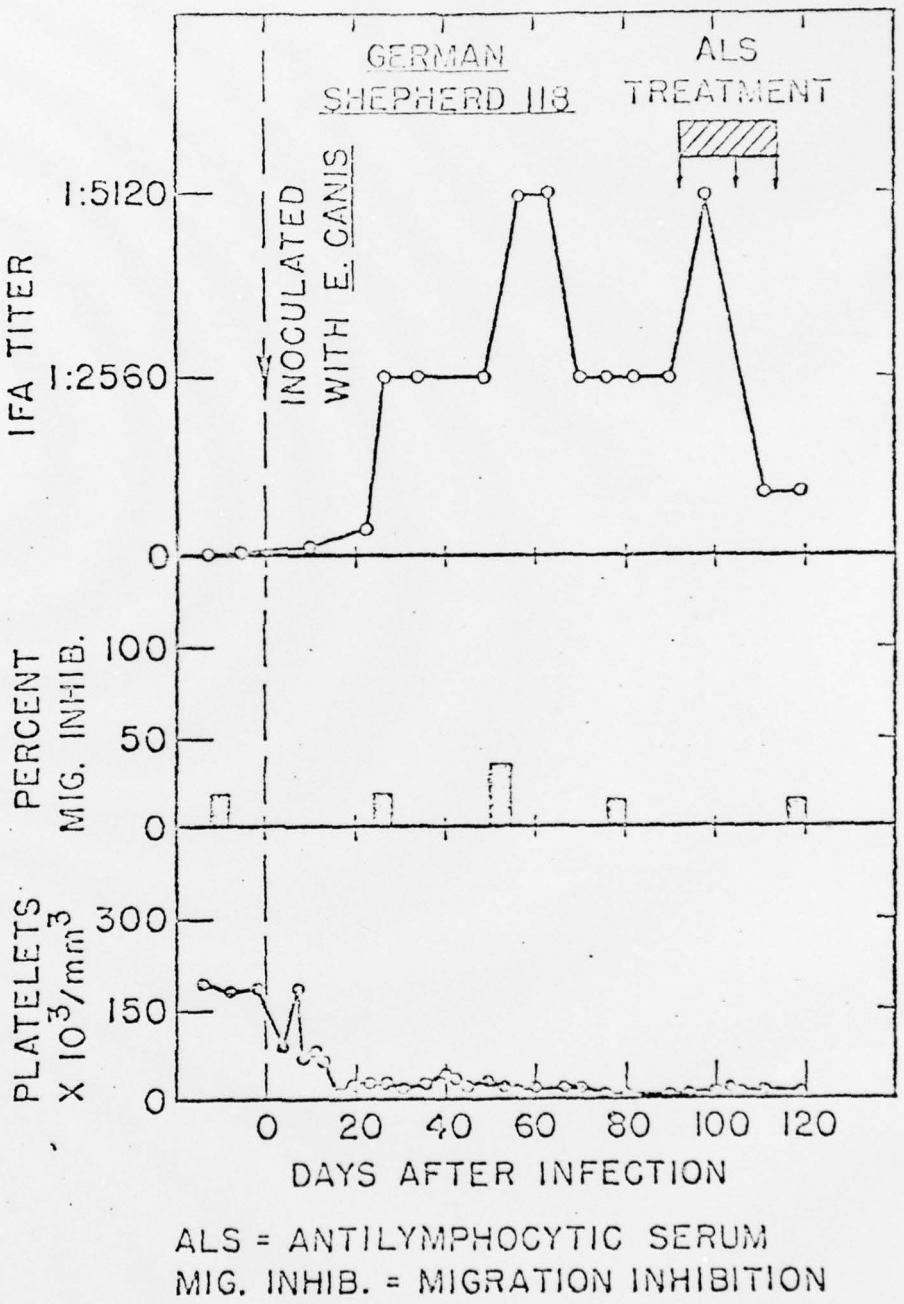


FIG. 6. Leukocyte migration inhibition test, indirect fluorescent antibody (IFA) titer, and thrombocyte counts for German shepherd dog No. 118 that had severe chronic TCP. The effect of treatment with antilymphocyte serum (ALS) is shown. Note that the thrombocyte counts for the dog were below 50,000/cmm 60 days post infection and that it was not influenced by treatment with ALS.

Figure 7

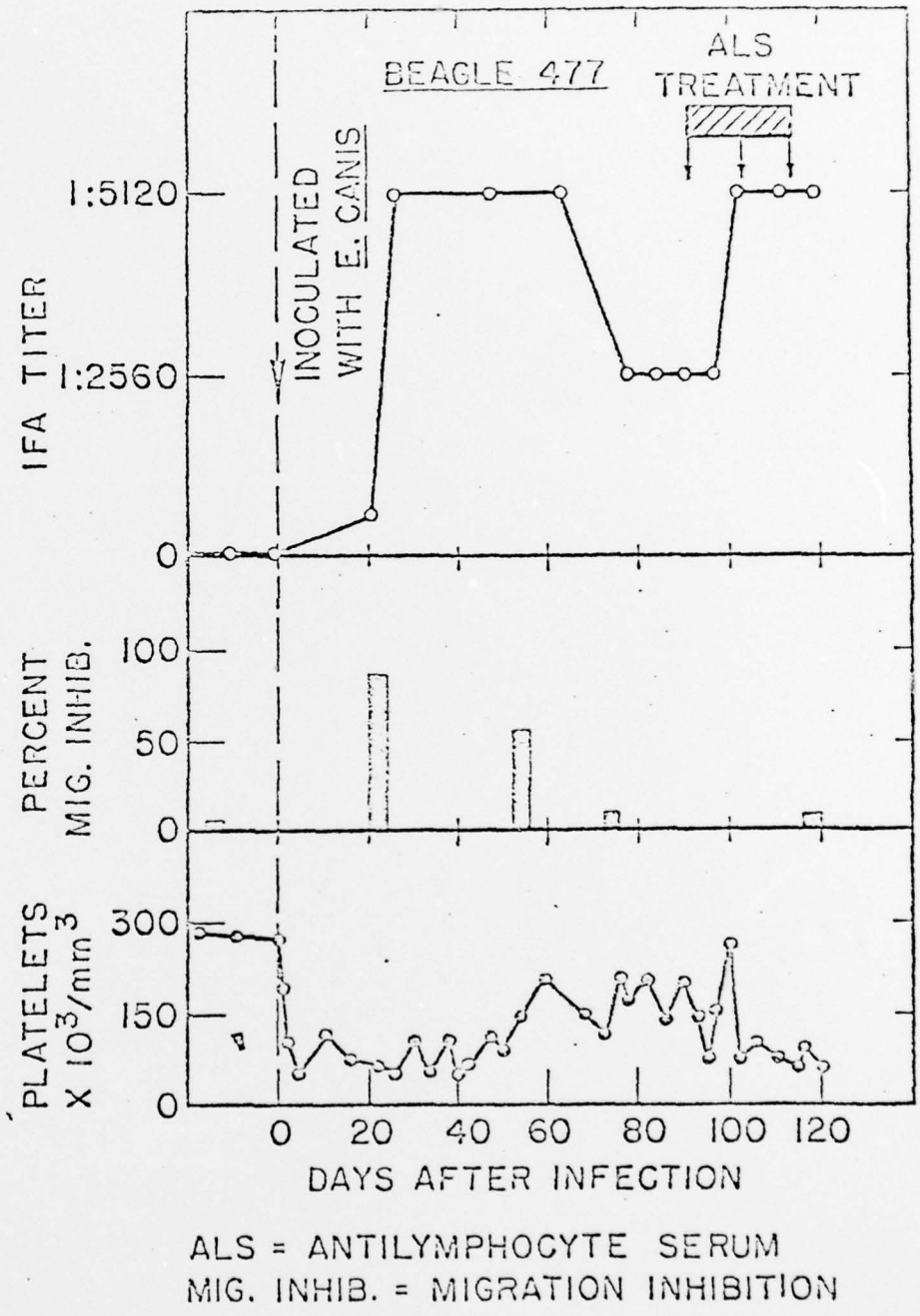


FIG. 7. Leukocyte migration inhibition test, indirect fluorescent antibody (IFA) titer, and thrombocyte counts for beagle dog No. 477 inoculated with Ehrlichia canis. Treatment with antilymphocyte serum (ALS) produced a transitory thrombocytopenia, interrupted by a transitory thrombocytosis.

Figure 8

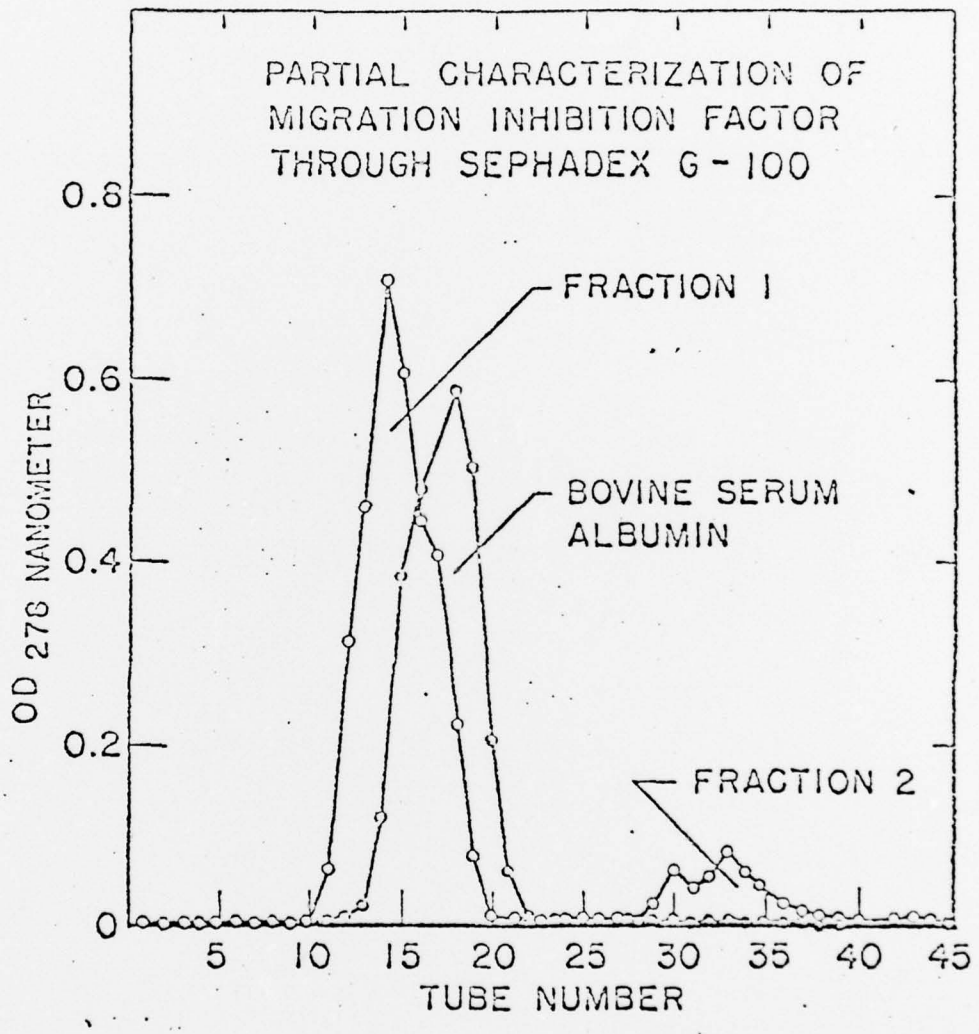
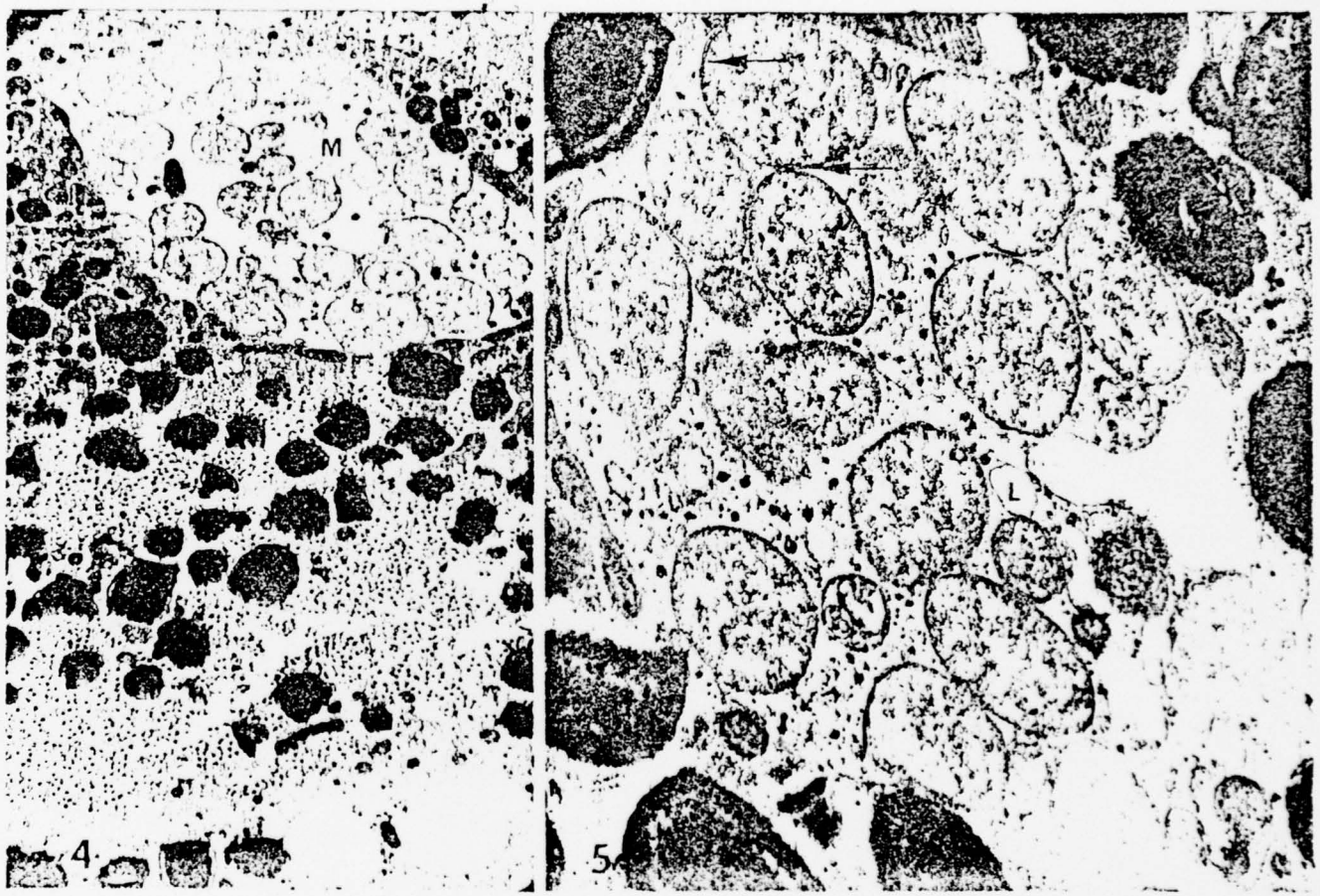
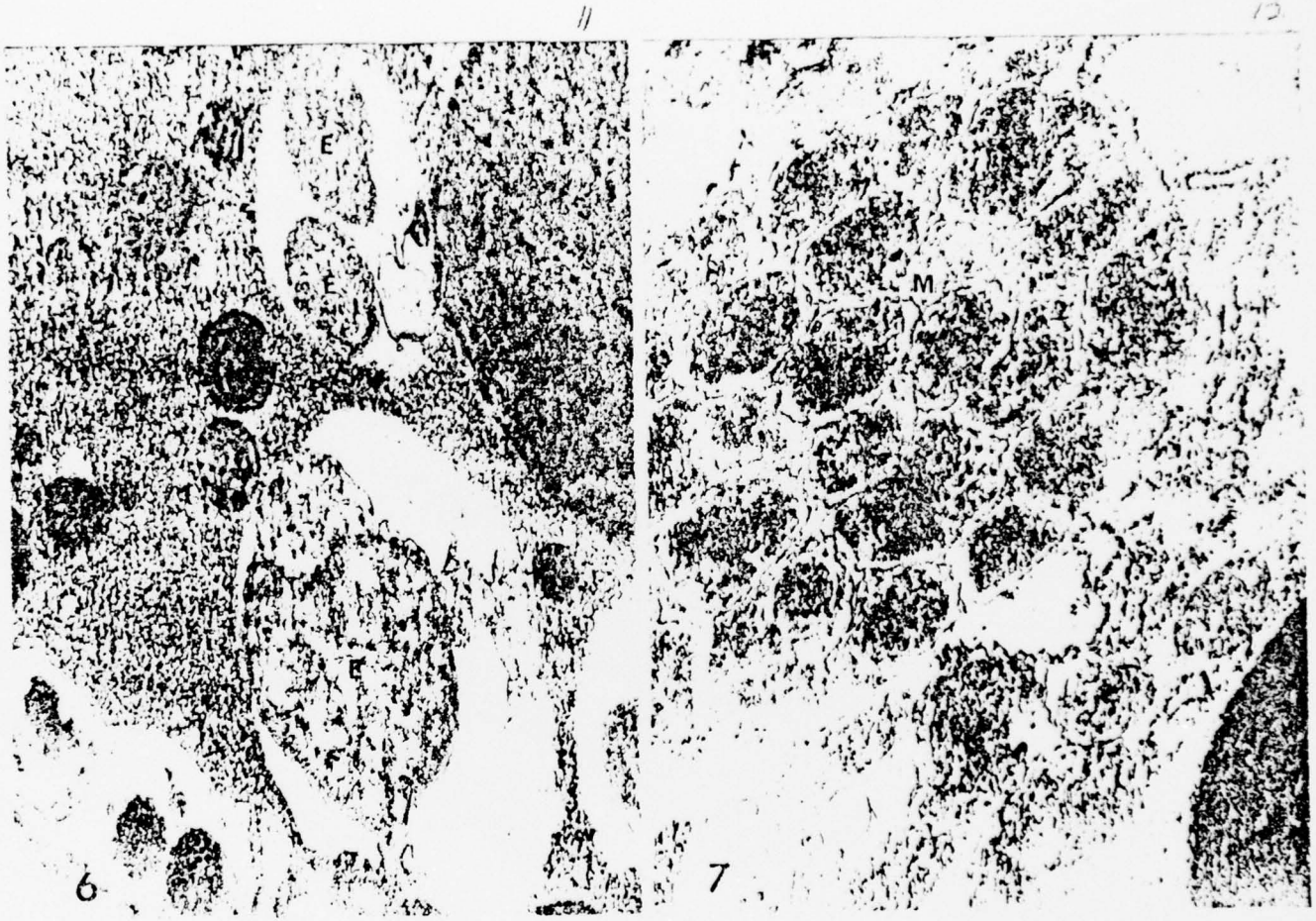


FIG. 8. Partial characterization of migration inhibition factor (MIF) from 4 Ehrlichia canis-infected beagle dogs through sephadex G-100. Migration inhibition factor was eluted at about the same level as bovine serum albumin (BSA).

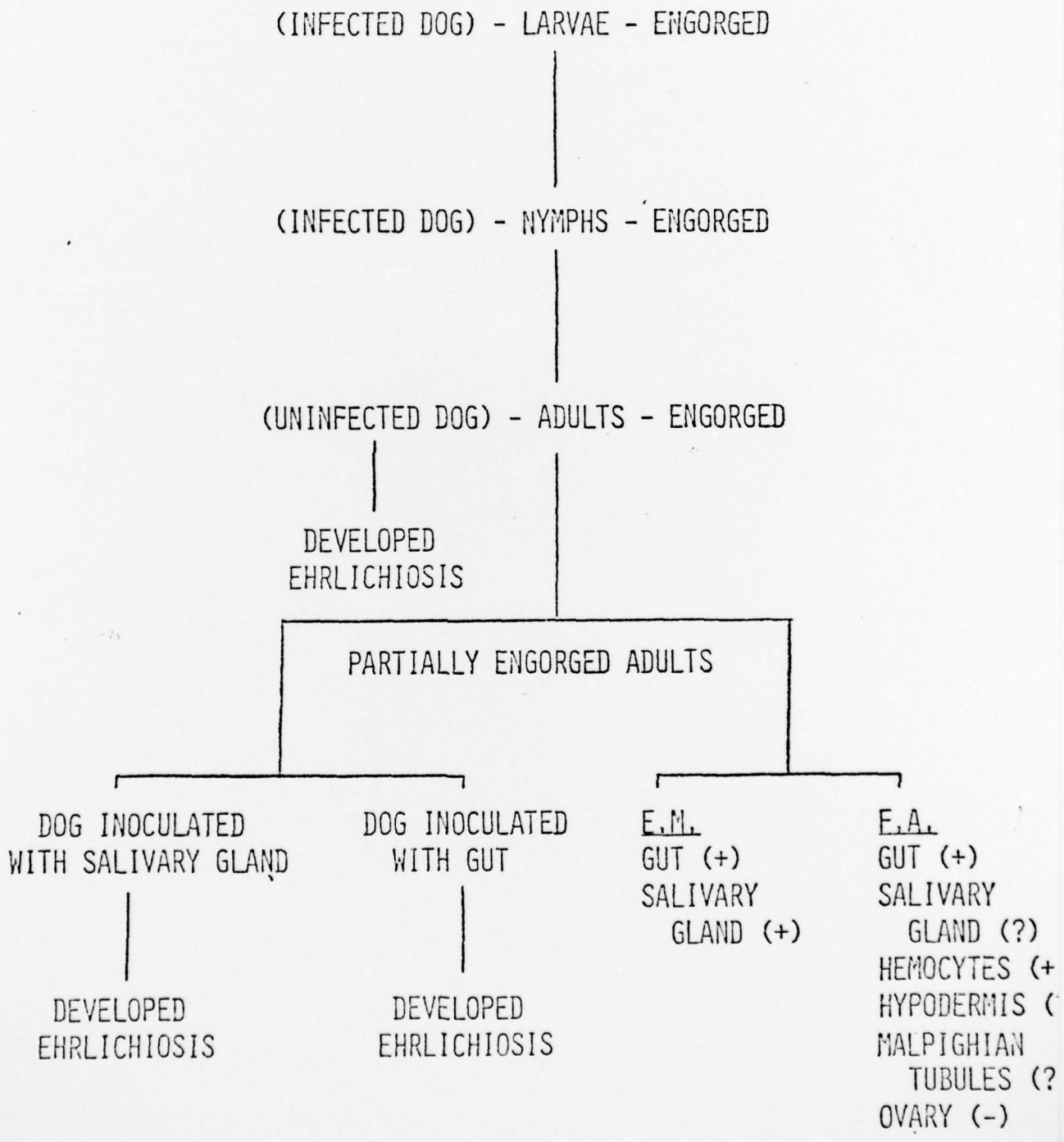


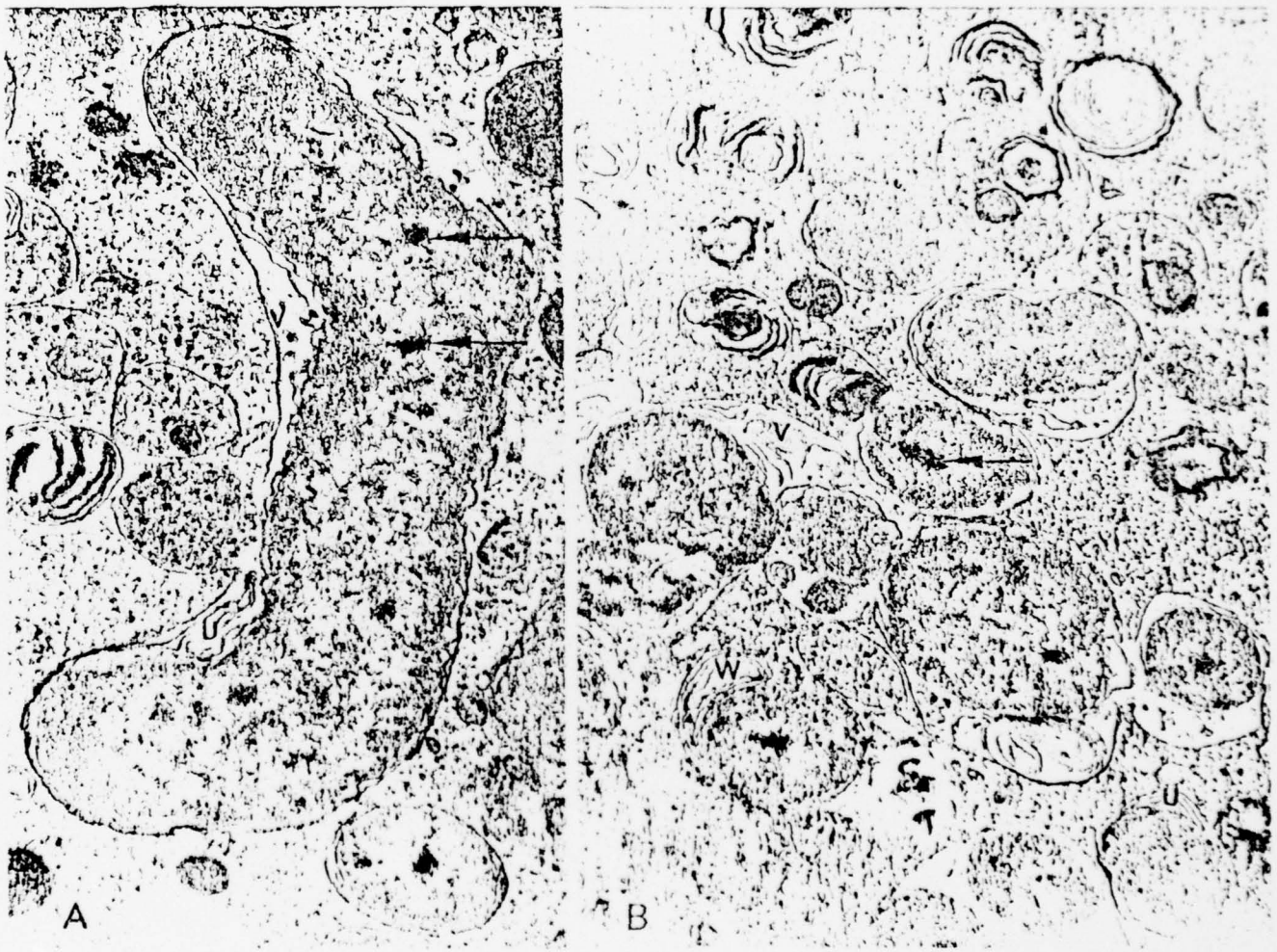
FIGS. 9 and 10. E. canis in mid-gut of epithelium of infected Rhipicephalus sanguineus.



FIGS. 11 and 12. Cytoplasmic vacuoles within salivary gland epithelial cell containing E. canis.

Figure 13


(INFECTED DOG) - LARVAE - ENGORGED
(INFECTED DOG) - NYMPHS - ENGORGED
(UNINFECTED DOG) - ADULTS - ENGORGED
DEVELOPED EHRLICHIOSIS
PARTIALLY ENGORGED ADULTS
DOG INOCULATED WITH SALIVARY GLAND
DOG INOCULATED WITH GUT
E.M.
GUT (+)
SALIVARY GLAND (+)
E.A.
GUT (+)
SALIVARY GLAND (?)
HEMOCYTES (+
HYPODERMIS (
MALPIGHIAN TUBULES (?
OVARY (-)
DEVELOPED EHRLICHIOSIS
DEVELOPED EHRLICHIOSIS



FIGS. 14 and 15. Symbiotic Rickettsia in tick ovary.

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